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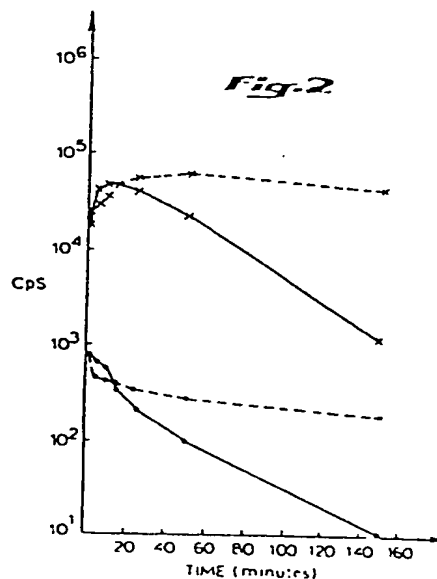
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⑤④ **Use of oxidase enzyme systems in chemiluminescent assays.**

⑤⑦ An oxidase enzyme system to provide long lived entities capable of being recognized by a chemiluminescent reagent is disclosed. In the preferred embodiments, a specific binding pair ligand or analyte is coupled with xanthine oxidase, either directly or via a streptavidin bridge. Thereafter the presence of an analyte can be determined by a chemiluminescent emission upon addition of a signal reagent comprising an oxidase substrate, a chemiluminescent reagent, such as luminol, and an enhancer, i.e. iron EDTA complex with a buffer providing a pH ranging from about 9.5 to about 10.5 The resulting chemiluminescent signal is stable and detectable for many hours or days after initiation. The chemiluminescent oxidase system is particularly useful for immunoassays and nucleotide probe analysis.



BACKGROUND OF THE INVENTION

This application claims benefit of a prior foreign filing made in France on August 6, 1986, Serial
5 No. 86-11415.

The present invention relates to the use of an xanthine oxidase (XO) enzyme reaction system in chemiluminescent assays, including immunoassays and DNA probe
10 analysis.

The use of bioluminescence and chemiluminescence in immunoanalysis has been extended to many analytes (KINGLER, W., STRASBURGER C.J., and WOOD W.G., Trends in
15 Analytical Chemistry 2:132-136, 1983; KRICKA L.J., and THORPE G.H.G., Analyst 108:1274-1296, 1983).

Chemiluminescent assays generally fall into two categories:

20

(a) The quantifiable phenomenon consists of a very brief signal (a few seconds) obtained after instantaneous injection of triggering reagent into the reaction medium. Such is the case with assays
25 using, as a tracer, coupling derivatives of luminol (SCHROEDER H.R., et al: Methods Enzymology 57:424-445, 1978) or of acridinium esters (WEEKS I. and WOODHEAD J.S. in Analytical Applications of Bioluminescence and Chemiluminescence, Academic Press,
30 pp. 185-188, 1984). Apart from the disadvantage associated with the brevity of the signal, this procedure demands the use of an apparatus for injecting the reagents into the measuring chamber itself. In consequence, application to micro-
35 titration plates is difficult and relatively

sophisticated and expensive apparatus is required for other detection vessels.

5 (b) The quantifiable phenomenon consists of a longer-lasting signal, which can be measured for a significant time period, enabling measurements to be repeated where appropriate and the samples to be prepared outside the luminometer. In such a system, an injection system is not required, thereby simplifying
10 the design of the luminometer and allowing use of microtitration plates.

At present, only one procedure has been described in which a relatively stable and intense chemi-
15 luminescence signal is obtained. This procedure is based on enhancement of the peroxidase-dependent oxidation reaction of cyclic diacylhydrazides by compounds such as synthetic luciferin, 6-hydroxybenzothiazole derivatives or substituted phenols (para-iodophenol) [WHITEHEAD T.P.,
20 THORPE G.H.G., CARTER T.J.N. et al., Nature (London) 305:158-159, 1983; THORPE G.H.G., KRICKA L.J. et al., Anal. Biochem. 145:97-100, 1985; THORPE G.H.G., KRICKA L.J. et al., Clin. Chem. 31:1335-1341, 1985]. This method is currently applied to the assay of many analytes.
25 However, it should be noted that the term and constancy of the signal is only relative, since the measurements can be performed only during some thirty or forty minutes.

The use of xanthine oxidase in a light producing
30 system has been previously contemplated by others, such as Boguslaski, U.S. Patent No. 4,363,759 and Molecular Biosystem, Inc., WO-A-85 03,356. However, no evidence or parameters are described which result in a long duration signal. Furthermore, xanthine oxidase is listed together
35 with many other enzyme systems which, as a whole, do not produce both H_2O_2 and superoxide anion radicals.

SUMMARY OF THE INVENTION

It has now been discovered that certain oxidase enzymes such as xanthine oxidase, choline oxidase, 5 sarcosine oxidase and fumarate oxidase, which produce oxygen-containing free radicals (e.g., superoxide anion $O_2^{\cdot -}$, hydroxyl radical OH^{\cdot}) and peroxide (H_2O_2 : hydrogen peroxide) provide long-lived chemiluminescent detectable products upon reaction with chemiluminescent reagents, 10 such as lucigenin, luminol and its derivatives. These chemiluminescent products provide a signal which is very stable with time and measurable during periods of several hours, even up to several days; this stability and long duration is much greater than that of all the methods 15 known at present.

The oxidase enzyme systems of the present invention are particularly useful as a tracer for detection of analytes in immunoassays, immunoblotting, or 20 nucleotide probe analysis to provide long lived light emitting entities after reaction with a chemiluminescent reagent. In the context of this invention, these long-lived light emitting entities are stable for more than 20 hours, with most emitting light for 120 hours and more.

25

In general, the present chemiluminescent oxidase systems provide a tracer consisting of analyte or ligand coupled to the selected oxidase enzyme. This tracer can be detected by a chemiluminescent reaction upon the 30 addition of a signal reagent. The signal reagent comprises an oxidase substrate; an enhancer, for instance a transition metal complex, particularly an iron complex; and a chemiluminescent reagent, dissolved in high ionic strength organic-based alkaline pH buffer, such as a 35 barbital, borate or carbonate buffer, and a preferred pH range of about 9.5 to about 10.5.

The substrates which are associated with the oxidase enzyme systems are any of the substrates specific for the selected enzyme. For example, when xanthine oxidase is selected, the substrate associated with xanthine oxidase (XO) in this enzyme system includes hypoxanthine, xanthine, acetaldehyde or purine. Hypoxanthine is most especially preferred.

In the context of the present invention, the chemiluminescent reagent is selected from acridinium esters, lucigenin or its derivatives, or luminol or its derivatives. Luminol and its derivatives are 2,3-dihydro 1,4-phthalazinedione compounds, which have been defined in several previous patents, e.g., U.S. Patent Nos. 4,598,044, 4,478,817 and 4,363,759. Acridinium esters, lucigenin and its derivatives have previously been defined in U.S. Patent No. 4,478,817.

Further, the present invention features different methods of assay employing the subject chemiluminescent oxidase enzyme systems.

The chemiluminescent oxidase enzyme systems of this invention are particularly useful in the context of immunoenzymatic analytical procedures such as immunometric or competitive binding assays. Such immunoassay techniques are generally well known in the art. For example, David et al., U.S. Patent No. 4,486,530, provides in its BACKGROUND OF THE INVENTION a discussion of various design techniques for enzyme linked immunoassays.

The present oxidase enzyme systems can also be employed in immunoassay procedures using the avidin biotin system. (See Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 15, Edited by R. H. Burdon et al. Elsevier Press.) In that case, a conjugate of enzyme-

biotin takes advantage of the high affinity constant of the avidin-biotin interaction to obtain a stable biotinylated enzyme-avidin stable complex. This complex provides a universal tracer which can be used in both
5 types of immuno analysis, immunometric and competitive. Indeed, it can be optimized to present free avidin sites (each molecule of avidin can fix four molecules of biotin), which can fix other biotinylated molecules.

10 Further, the chemiluminescent oxidase systems of the present invention are useful as a chemiluminescent detection system for nucleotide probe analysis. A general discussion for nucleotide probe hybridization techniques is given in Falkow et al., U.S. Patent No. 4,358,535.

15 Thus, in general, the chemiluminescent oxidase systems of the present invention can be adapted for use in a wide variety of assays designed to detect an analyte wherein a specific binding pair ligand is coupled with an
20 enzymatic or substrate tracer.

Consistent with the generally accepted terminology in the art and in the context of this disclosure, the following terms are defined:

25 As used herein, an analyte is a specific bindable material whose presence or amount is to be determined. Examples of analytes include antigens, haptens, antibodies, glycosylated proteins, carbohydrates
30 and oligonucleotides.

As used herein, a ligand is a specific binding partner of the analyte, which has a specific binding affinity for the analyte to the exclusion of other
35 substances. Examples of ligands include antibodies, lectins and oligonucleotides.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following discussion is in terms of the preferred embodiments of this invention, which represent
5 the best mode known to the Applicant at the time of this application.

The present invention is based on the realization that certain oxidases which produce both H_2O_2
10 and $O_2^{\cdot-}$, including, for example, xanthine oxidase, choline oxidase, fumarate oxidase and sarcosine oxidase, are capable of generating long-lived light emitting entities when reacted with an oxidase substrate, a chemiluminescent reagent and an enhancer under pH conditions ranging from
15 about 9.5 to about 10.5.

Specific examples of design methodologies of using the chemiluminescent oxidase enzyme systems of the present invention include the following:

20

In a method for the detection of a hapten or antigen as the analyte, a competitive immunoassay can be employed comprising the steps of:

25

(a) providing a solid support such as a tube, microtiter well, or beads, deposited with a ligand such as a lectin or antibody specific for said analyte;

30

(b) providing a tracer consisting of an analyte coupled to an oxidase by direct covalent linkage or by an avidin bridge between biotinylated enzyme and biotinylated analyte;

35

(c) incubating the sample containing the analyte, antigen or hapten to be assayed in the

presence of the solid support prepared in stage
(a) and the tracer prepared in stage (b);

(d) washing the support;

5

(e) adding a signal reagent which includes
a substrate associated with the oxidase, an
enhancer and a chemiluminescent reagent, main-
tained at a pH ranging from about 9.5 to about
10.5; and

10

(f) measuring the chemiluminescent response
and deducing the concentration of antigen or
hapten from the measurement thereby performed.

15

The kit of reagents required for carrying out
this method, which also forms the subject of the present
invention, is characterized in that it contains:

20

(a) a solid support, such as a tube, micro-
titer plate, or beads, coated with ligand such as
an antibody or lectin specific for the antigen or
hapten analyte to be assayed;

25

(b) a product of coupling of the analyte
and an oxidase; and

30

(c) a substrate associated with the
oxidase, a chemiluminescent reagent, and an
enhancer, all maintained at a pH ranging from
about 9.5 to about 10.5.

35

In a method to detect an antigen or antibody as
the analyte, an immunometric assay can be employed
comprising the steps of:

(a) providing a solid support such as a tube, microtiter well, or beads, deposited with a ligand such as a lectin or antibody specific for said analyte;

5

(b) providing a tracer consisting of a complementary ligand coupled to an oxidase by direct covalent linkage or by an avidin bridge between biotinylated enzyme and biotinylated ligand;

10

(c) incubating a sample containing the analyte to be assayed in the presence of the solid support and the tracer;

15

(d) separating the unbound tracer from the bound tracer by washing the solid support;

20

(e) adding to the washed support a signal reagent comprising an oxidase substrate, a chemiluminescent reagent and an enhancer maintained at a pH ranging from about 9.5 to about 10.5; and

25

(f) measuring the chemiluminescent response and deducing the amount of the analyte in the sample.

A kit of reagents for carrying out an immunometric chemiluminescent assay of the present invention would include:

30

(a) a solid support, such as a tube, microtiter plate, or beads, coated with a ligand such as an antibody or ligand specific for the analyte to be assayed;

35

(b) a tracer consisting of a coupling product of an oxidase and a second ligand which is specific for the analyte to be assayed; and

5 (c) a signal reagent comprising an oxidase substrate, a chemiluminescent reagent, and an enhancer, all at a pH ranging from about 9.5 to about 10.5.

10 With respect to nucleotide probes hybridization techniques, the present chemiluminescent oxidase system can be used to detect a hybridized duplex formation as follows:

15 (a) depositing genetic material of an analyte to a solid support, such as a filter, tube or microtiter plate;

20 (b) incubating under hybridizing conditions a tracer consisting of an oligonucleotide probe coupled to an oxidase, the probe having a nucleotide sequence substantially complementary to the genetic material of the analyte;

25 (c) washing the support;

30 (d) adding a substrate associated with the oxidase, an enhancer and a chemiluminescent reagent under pH conditions ranging from about 9.5 to about 10.5; and

35 (e) measuring the chemiluminescent response to determine the concentration or presence of the duplex formed.

A kit of reagents for carrying out this method includes:

5 (a) a solid support, such as a filter, tube or microtiter plate;

(b) an oligonucleotide probe coupled with an oxidase; and

10 (c) a substrate associated with the oxidase, a chemiluminescent reagent, and an enhancer, all maintained at a pH ranging from about 9.5 to about 10.5.

15 Finally, of course, the invention can be used in an assay to detect an oxidase in biological samples. For example, such an assay involves:

20 (a) providing a solid support, such as a tube, coated with an antibody to oxidase;

(b) incubating the sample containing the oxidase to be assayed in the presence of the solid support prepared in stage (a);

25 (c) washing the support;

30 (d) adding to the support prepared in stage (c) a substrate associated with the oxidase, an enhancer, and a chemiluminescent reagent maintained at a pH of about 9.5 to about 10.5; and

35 (e) measuring the chemiluminescent response and deducing the concentration of the oxidase from the measurement thereby performed.

A kit of reagents for carrying out this method, which also forms the subject of the present invention comprises:

5 (a) a solid support, such as a tube, coated with an antibody possessing specificity for an oxidase; and

10 (b) a substrate associated with the oxidase, a chemiluminescent reagent, and an enhancer, maintained at a pH from about 9.5 to about 10.5.

As a chemiluminescent reagent, luminol, iso-
15 luminol, N-(4-aminobutyl)-N-ethyl isoluminol, or lucigenin can be chosen; preferably, luminol is chosen.

In examples which follow, the advantages and applications of the present oxidase enzyme system in
20 immunoanalysis is illustrated on the basis of actual experimental results.

EXAMPLE 1: Assay of Xanthine Oxidase
in Liquid Phase

25

An example of an optimized procedure for assaying xanthine oxidase (XO) activity in liquid phase is described below.

30 a) Procedure

The sample xanthine oxidase to be analyzed (50
μl), was diluted in 0.1M sodium barbital solution containing bovine albumin (0.2%) and ethylenedia-
35 mineteraacetic acid, disodium salt (EDTA) (1 mM), and

mixed with 50 μ l of a solution₄ of hypoxanthine (10 mM) substrate and luminol (0.5×10^{-4} M) in distilled water.

The light measurement was then performed in front of the photomultiplier of a luminometer (PICOLITE 6500™ Packard Instruments Co.). The emission of photons was measured for a period of one second at room temperature. The results obtained are expressed as counts/second after subtracting the background (counts/second corresponding to the emission of light by the medium in the absence of xanthine oxidase).

This procedure was characterized by the extreme ease and extreme rapidity with which it was carried out, as well as by the possibility of automating the counting therein.

b) Kinetics of the Reaction

The main value of this reaction resides in the great stability and duration of the chemiluminescent signal obtained.

The kinetics of the xanthine oxidase-dependent oxidation of luminol for different enzyme samples are shown in Figure 1. The experimental conditions are those described above. The measurements for a period of one second, at intervals over a period of eight hours, demonstrate the stability of the signal. It should be noted that, for xanthine oxidase samples less than 1 femtomole (fmol), complete stabilization of the emission is attained 10 minutes after the start of the reaction. On the other hand, for higher concentrations in the range 10 to 100 fmol per sample, the signal gradually increases and stabilization is obtained in one hour. In point of fact,

these variations of light output remain negligible with respect to the measurement time.

5 Comparing the kinetics of this chemiluminescent response with that corresponding to the other stable systems which have been described at the present time demonstrate the value of xanthine oxidase in immuno-analysis: The measurements can be repeated without loss of sensitivity for a very extended period of time.

10

Other results have shown that the signal can be measured for periods of at least 20 hours.

15 The stability of the emission of photons can be interpreted in accordance with the catalytic nature of the xanthine oxidase reaction, the stability being linked to the gradual accumulation of H_2O_2 originating from the dismutation of $O_2^{\cdot -}$. This sequence then brings into play the HABER-WEISS reaction which is responsible for the
20 formation of hydroxyl radicals and the subsequent oxidation of luminol generating light. Henry J. P., Michelson A.M. (1977) in Superoxide and Superoxide Dismutases - Ed. by Michelson A.M., McCord J. and Fridovich I. pp. 283-290, Academic Press.

25

GENERAL CHARACTERISTICS OF THE CHEMILUMINESCENT REACTION

Apart from the stability of the signal, the
30 general characteristics of the XO reaction are as follows:

a) Influence of the Molarity of the Medium

35 Chemiluminescent response obtained for variable concentrations of barbital between 0.025M and 0.25M demonstrated that the stronger the molarity, the greater the

responses. A final barbital concentration of 0.25M was consequently adopted for the routine procedure.

b) Influence of the pH of the Medium

5

The chemiluminescent responses of the XO system was optimized with the use of alkaline range buffers. Use of buffers with pH ranging from about 9.5 to about 10.5 yielded increases in light production of 10-100 times
10 compared to lower pH's. Useful buffers include phosphate, borate, carbonate, barbital and TRIS.

c) Influence of the Nature of the Chemiluminescent Compound

15

Luminol does not represent the only substance whose oxidation leads to the emission of photons in the XO system. This property is also shared by lucigenin (N-methyl acridinium).

20

An experiment determined the respective concentrations of lucigenin (10^{-5} M) and luminol (0.5×10^{-4} M) giving the greatest chemiluminescent response for an XO sample of 23 ng (77 femtomoles).

25

The use of luminol provided much greater sensitivity (< 0.3 femtomoles) than that obtained with lucigenin. This fact is probably linked to the special reactivity of luminol with respect to oxygen-containing
30 radicals produced by the XO reaction (O_2^- , H_2O_2 , OH^\cdot), whereas lucigenin is more specific for the superoxide anion. In this case, the information contributed by the hydrogen peroxide and the hydroxyl radicals is not taken into account.

35

The use of luminol at a final concentration of 0.5×10^{-4} M) was hence adopted for the routine xanthine oxidase assay procedure.

5 d) Influence of the Nature of the Substrate

Figure 2 shows the comparison of the curves for light emission in terms of time during the xanthine oxidase-dependent oxidation of luminol, with two different substrates, hypoxanthine and acetaldehyde, and with two
10 different concentrations of xanthine oxidase.

Legend of Figure 2:

15 ----- 5mM hypoxanthine
 _____ 50 mM acetaldehyde
 X-----X 80 femtomoles XO
 20 _____ 0.8 femtomole XO

The stability of the signal obtained with hypoxanthine was much more pronounced than that which
25 corresponds to acetaldehyde. In this latter case, in effect, the decrease occurs after an interval of 20 minutes following the initiation of the reaction.

After 150 minutes, the intensity of the signal
30 with acetaldehyde represents only 4% of the maximal response achieved with hypoxanthine. In the case of hypoxanthine, no decrease was observed at this point in time. This justifies the routine use of hypoxanthine as a substrate for obtaining an emission of photons which is
35 both stable and high.

e) Potentialiation of the XO-Dependent Oxidation of Luminol by the Iron/EDTA Complex

It is generally accepted (WINTERBOURN C.C. et al, Archives of Biochemistry and Biophysics, 244, 1, 27-34, 1986) that the iron/ethylenediaminetetra-acetic acid (EDTA) complex represents an efficient catalyst for the HABER-WEISS reaction according to the following mechanism:

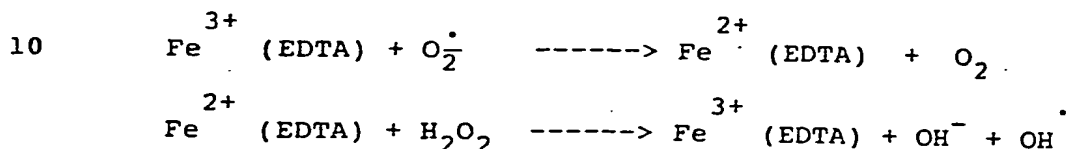


Figure 3 shows the chemiluminescence curves measured 15 minutes after triggering the reaction with XO and obtained in the presence or absence of iron/EDTA complex.

Legend of Figure 3:

20

. _____ . 5 μ M EDTA-Fe
 x _____ x 0 μ M EDTA-Fe

The iron complex was prepared by mixing equal parts of FeCl_2 (1mM) and EDTA-Na_2 (100 mM) solution, both prepared in distilled water. After a minimum time of 30 minutes elapsed, this solution was diluted 1/100 in the hypoxanthine/luminol mixture. The final concentration of the complex in the incubation medium for the XO reaction is hence 5 μ M.

The EDTA/iron complex increases by a factor of 10 the chemiluminescent response linked to the XO-induced oxidation of luminol. The use of the complex hence enables the threshold of detection of the XO activity to be lowered.

Figure 4 shows, furthermore, that the signal produced in the presence of this complex was slightly less stable than that obtained in its absence; however, 7 hours 30 minutes after initiation of the reaction, the emission of photons obtained and measured as chemiluminescence in the presence of Fe/EDTA remained much higher than that observed in its absence.

Legend of Figure 4:

10

Kinetics of the xanthine oxidase-dependent oxidation of luminol in the presence X_____X or absence _____ of EDTA/Fe complex (5 μ M), for concentrations of xanthine oxidase of 0.8 and 80 femtomoles.

15

These properties show that the iron/EDTA complex represents a good amplification agent for the reaction in question and that its use is recommended in the routine assay procedure.

20

EXAMPLE 2: Solid Phase Assay of Avidin

1. Preparation of the XO-Biotin Conjugate

25

A suspension of XO in 3.2M $(\text{NH}_4)_2\text{SO}_4$ (20 mg/ml) (Boehringer Mannheim) was centrifuged at 5000g during 10 minutes and the pellet was dissolved in 1 ml pH 8.5 50 mM, NaCl, 20 mM borate buffer. A solution of biotin X NHS (Biotin ϵ -aminocaproic acid N hydroxysuccinimide ester, Calbiochem) (1.2mg/12 μ l anhydrous dimethylformamide) was added. The solution was agitated by vortexing for 2 minutes, left at ambient temperature for one hour, and then dialyzed against 0.13M NaCl, 0.07M Na phosphate buffer pH 7.4 (Phosphate buffered saline PBS) containing

35

0.1% Na azide. The XO-biotin conjugate was then kept at +4°C under sterile conditions.

2. Bovine Serum Albumin Biotin Conjugate

5 50mg bovine serum albumin (BSA) (Fraction V, Fluka) were dissolved in pH 8.5 50 mM NaCl, 20 mM borate buffer. A solution of biotin X NHS (20 mg/200 µl anhydrous dimethylformamide) was then added, and the
10 following steps were identical to those described for the preparation of biotinylated XO.

3. BSA-Biotin Coated Tubes

15 150 µl of biotinylated BSA (5 µg/ml) in pH 9.6 0.1M carbonate buffer were added to polystyrene tubes (SARSTEDT, 3.5ml, 55 x 12 mm). The tubes were incubated at +4°C for 16 hours, then washed twice with distilled water and the remaining protein binding sites of the tube
20 were saturated with a 0.3% solution of BSA (500 µl) in distilled water containing 0.1% sodium azide.

4. Avidin Assay

25 BSA biotin coated tubes were washed twice with 1ml of phosphate buffered saline (PBS) containing 0.1% BSA, then 200 µl of a solution containing various concentrations of egg avidin (Calbiochem) were added. The tubes were incubated 30 minutes at ambient temperature.

30 After washing the tubes twice with PBS containing 0.1% BSA (PBS BSA), the XO biotin (2 µg/200 ml PBS BSA) was added and the tubes were again incubated 2 hours at ambient temperature. The tubes were washed, then the
35 signal reagent (200 µl) was added. The composition of the signal reagent was the following:

0.5 x 10⁻⁴ M luminol
 0.25 M Na barbital buffer pH 10.2
 5 mM hypoxanthine
 0.25 mM Na₂ EDTA
 5 μM Cl₂ Fe

The tubes were placed in the luminometer and the counting was performed for five seconds. Figure 5 shows a representative example of an avidin assay performed with this protocol.

Legend of Figure 5:

Abcissa: Concentration of avidin in μg/ml (log scale).

Ordinate: Ratio of counts per second in presence and in absence of avidin (log scale).

It was thus demonstrated that the magnitude of the light intensity produced by the XO dependent chemiluminescent system was a function of the quantity of avidin present in the specific binding reaction mixture. The present invention thus provides conjugates (biotinylated - XO) useful for the determination of ligands in a liquid medium by a solid phase assay.

EXAMPLE 3: Solid Phase Competitive Biotin Assay

The steps of the biotin competitive binding assay were as follows:

A preformed complex biotinylated XO (10 μg/ml) - Streptavidin (0.3 μg/ml) was prepared in PBS BSA, and incubated 30 minutes at ambient temperature. This was

added to BSA-biotin coated tubes, prepared as previously described:

-100 μ l of a solution containing various concentrations of biotin.

-100 μ l of the preformed complex.

Afterward the tubes were incubated for two hours at ambient temperature (or overnight at +4°C). Then the tubes were washed twice with 1ml of PBS BSA. The chemiluminescent signal reagent (200 μ l) was then added and the tubes counted in the luminometer during five seconds.

Figure 6 shows a representative example of a competitive assay performed with this protocol. The sensitivity of this assay is about 5 pg (20 femtomoles).

Legend of Figure 6:

Abscissa: Concentration of biotin in pg per sample (linear scale).

Ordinate: Counts per second (linear scale).

The magnitude of the light intensity produced by the XO dependent chemiluminescent system was an inverse function of the quantity of biotin in the specific reaction mixture. The present invention thus provides complexes useful as tracer for the determination of ligands in a liquid medium by a competitive solid phase assay.

EXAMPLE 4: Detection of Toxoplasma Antibodies

a) Preparation of Biotinylated Antihuman IgG

5 0.71 ml of a 2.8 mg/ml rabbit antibody to human
IgG solution in 0.25M NaCl, 0.02M phosphate pH 7.6 buffer
(Jackson Laboratories) was added to 8 µl of a solution of
Biotin X NHS (10 mg/100 µl dimethylformamide). The
following protocol was then identical to that described
10 for the biotinylation of XO.

b) Toxoplasma Antigen Coated Tubes

 The Toxoplasma antigen preparation had been
15 purchased from Institute Virion (Zurich, Switzerland) and
reconstituted according to the recommended instructions
provided. Polystyrene tubes were coated with 150 µl of
reconstituted antigen preparation, and incubated overnight
at ambient temperature. They were kept dry at -20°C after
20 washing with PBS BSA buffer.

c) Preformed Biotinylated XO - Streptavidin -
Biotinylated Anti IgG Complex

25 A complex biotinylated XO - streptavidin -
biotinylated rabbit IgG was prepared in PBS BSA by
incubating biotinylated XO (10 µg/ml) with streptavidin
(0.5 µg/ml) for 15 minutes at ambient temperature, then
adding biotinylated rabbit IgG (final concentrations: 1.4
30 µg/ml).

d) AntiToxoplasma Assay

 The steps of the Toxoplasma antibody assay were
35 as follows:

Coated tubes were incubated with 200 μ l of positive or negative control serum for one hour at -37°C . The Toxoplasma control sera were purchased from Biotrol Laboratories. The tubes were then washed with PBS BSA buffer and preformed complex (200 μ l) added.

The tubes were incubated three hours at ambient temperature (or overnight at 4°C), and then washed twice with PBS BSA. Afterward, 200 μ l of the chemiluminescent signal reagent was added as described above and the tubes counted in luminometer for five seconds.

An example of the kinetics of the chemiluminescent signal, obtained with two different dilutions of a Toxoplasma positive control serum, is shown in Figure 7. The maximal signal appeared within 30 minutes following the addition of the signal reagent, the signal decreases with a half life of 30 hours.

Legend of Figure 7

Abcissa: Time (hours) (linear scale)

Ordinate: Counts/second (logarithmic scale)

Figure 8 shows the chemiluminescent responses obtained with serial dilutions of both positive and negative control sera.

Legend of Figure 8

Abcissa: Microliters, control serum
(logarithmic scale)

Ordinate: Ratio of counts per second in presence and in absence of diluted sera (logarithmic scale)

5 The intensity of a chemiluminescent signal produced by a complex [biotinylated XO - Streptavidin - biotinylated anti human IgG antibody] which is immobilized on a solid phase, itself coated with the corresponding antigen, was directly related to the quantity of human IgG
10 to Toxoplasma in the sample to be analyzed. Further, the obtained signal presented a long time emission, on the order of days.

Similar results were obtained for an assay
15 designed in the same manner as the Toxoplasma antibody assay, and allowing the detection of Chlamydiae antibody in human serum.

EXAMPLE 5: Prolactin Immunoassay

20

Two monoclonal antibodies directed at separate epitopes of the prolactin molecule used in this immunoassay were a generous gift of Immunotech (Luminy, France).

25 a) Preparation of Biotinylated Monoclonal Antiprolactin Antibody

1 mg of IgG₁ monoclonal antiprolactin antibody in 0.75 ml of 0.15mM NaCl, 20 mM borate, pH 8.6 buffer was
30 treated with 40 µg of biotin X NHS dissolved in 20 µl of dimethylformamide. The remaining protocol was then identical to that described above for the preparation of biotinylated XO.

b) Antiprolactin Antibody Coated Tubes

Polystyrene tubes were coated with 19.5 µg of an IgG₂ a monoclonal antibody in 150 µl 0.1M carbonate buffer, pH 9.6. The coating was performed overnight at ambient temperature, then the tubes were washed once with distilled water, and the residual protein binding sites were saturated with 500 µl of a 0.3% BSA solution in distilled water. The coated tubes were kept at + 4°C.

10

c) Prolactin Immunoassay

The steps of the prolactin immunoassay were performed as follows:

15

- Incubating the coated tubes with 150 µl of anti-prolactin biotinylated antibody diluted in PBS BSA (10^{-2} dilution) and 50 µl of serum overnight at ambient temperature.

20

- Washing the tubes twice with PBS BSA.

25

- Adding 200 µl of the preformed complex: Biotinylated XO (10 µg/ml), Streptavidin (0.5 µg/ml) prepared in PBS BSA and incubating two hours at ambient temperature.

30

- Washing the tubes twice with PBS BSA.
- Adding 200 µl signal reagent.
- Counting each tube in the luminometer for five seconds.

35

The half life of the chemiluminescent signal corresponding to the concentration of 200 µg/ml was 30

hours. The comparison of the standard curves obtained 30 minutes, 21 hours, and 140 hours after the addition of signal reagent showed that the standard curves resulting from determining the chemiluminescence over this time course were essentially equivalent. The sensitivity appeared to improve with time because of the proportionately more rapid decay of the background. (Figure 9)

Legend of Figure 9

10

Abscissa: ng/ml prolactin (linear scale)

15

Ordinate: Ratio of counts per second obtained with the different standard sera to that obtained with the maximal concentration (200 ng/ml) (linear scale)

This thus demonstrated that the magnitude of the chemiluminescent signal produced by a complex [biotinylated XO - Streptavidin - biotinylated monoclonal antibody], immobilized by the way of the antigen on a solid phase, which is itself coated with a complementary monoclonal antibody, is directly related to the quantity of antigen in the sample. Also, the obtained signal demonstrated a long-lived emission, which can be expressed in terms of days.

EXAMPLE 6: Triiodothyronine (T3) Immunoassay

30

a) Preparation of the Tracer

1.19 mg of biotinylated BSA was diluted in pH 9.6, 0.1M carbonate buffer (3ml). 12 ml of 0.04M barbital buffer pH 9.4 are added, then 2.5 mg of T3, previously dissolved in 1ml dimethylformamide. The coupling reaction was initiated by adding 50 μ l of 0.04M glutaraldehyde in

distilled water. The mixture was incubated for three hours at ambient temperature, then the reaction was stopped by the addition of 200 μ l 0.1M glycine in distilled water. After an additional hour incubation, 100 μ l of a 37.5mM sodium borohydride solution were added. The mixture was kept at 4°C for 1.5 hours. The solution was filtration-concentrated to 1.5ml (Amicon B15) and then the conjugate was purified by gel filtration column chromatography (Ultrogel Aca 44, Industrie Biologique Francaise) (Size of the column: 60 x 0.9cm). The immunoreactive and enzymatically active fractions were respectively located by radioimmunoassay and by chemiluminescence in the hypoxanthine, luminol system. These fractions were pooled.

15

b) Coated Tubes

Polystyrene tubes were coated with 150 μ l 1.95 μ g/150 μ l of a goat antirabbit IgG (ARGG) (Jackson Laboratories) in 0.1M carbonate buffer pH 9.6. The incubation was performed overnight at ambient temperature, then the tubes were washed and the unreacted protein binding sites were saturated with 500 μ l of a 0.3% BSA solution in distilled water. These coated tubes were kept at +4°C.

25

c) Anti T3 Antibody

Anti T3 antibody was obtained by immunizing a rabbit with a T3 BSA conjugate derivatized with carbodiimide. The immunoglobulin was purified with 30% $(\text{NH}_4)_2\text{SO}_4$ precipitation, and then by DEAE Sephacel (Pharmacia, Uppsala, Sweden) chromatography.

30

d) T3 Immunoassay

The steps of the T3 assay were as follows:

- 5 - Incubating (3 hours, ambient temperature) ARGG
coated tubes with various concentrations of T3
(0 to 12.4 p moles), a fixed concentration of T3
biotinylated BSA conjugate, and anti T3 antibody
(final dilution: 1/15000). All dilutions are
10 performed in PBS BSA. The final volume of
incubation is 300 μ l
- 15 - Washing the tubes with PBS BSA, containing 0.05%
Triton X100
- 20 - Incubating (2 hours, ambient temperature) the
tubes with the preformed complex biotinylated XO
(10 μ g/ml), -Streptavidin (0.5 μ g/ml).
- 25 - Washing the tubes with PBS BSA.
- 30 - Adding 200 μ l of the signal reagent.
- 35 - Counting each tube in the luminometer for 5
seconds.

Table I shows the results obtained with two
different dilutions (10^{-3} , 10^{-4}) of the T3 biotinylated
BSA conjugate. CPS means counts per second; B/Bo means
30 CPS bound at concentration B divided by CPS bound in
absence of T3.

T3 pmoles	T ₃ Biotinylated BSA			
	10 ⁻³		10 ⁻⁴	
	CPS	B/Bo	CPS	B/Bo
0	70810	1.0	51500	1.0
0.124	48010	0.68	6530	0.13
1.24	11100	0.16	1080	0.02
12.4	1020	0.01	850	0.01

10

The intensity of the chemiluminescent signal produced by a complex [biotinylated XO - Streptavidin - hapten-biotinylated BSA], immunologically immobilized on a solid place was inversely related to the quantity of hapten in the sample.

15

EXAMPLE 7: Triiodothyronine Immunoassay Using T3-XO Conjugate

20

An example of application of the chemiluminescent system in immunoanalysis using hapten XO conjugate is provided by the competitive immunoassay of triiodothyronine.

25

a) Preparation of the T3-XO Conjugate

The coupling of T3 (Fluka) with a preparation of XO) (BOEHRINGER) was performed according to the method of YAMAMOTO, Clinical Chemistry, 27-10, pages 1721-1723, 1981.

30

XO was equilibrated in 0.1M phosphate buffer, pH 7, by passage through a Sephadex G-25 column (Pharmacia PD-10) in two successive chromatographic runs. The ratio of the absorbancies at 280 nm and 450 nm of the preparation obtained equaled 5.8 +/- 0.2.

35

600 µl of a solution of T3 containing 1 mg/ml in dimethylformamide (DMF) was treated with 60 µl of a

solution of 4-(maleimidomethyl) cyclohexane-1 carboxylic acid succinimide ester (SMCC) in DMF (2 mg/ml), and 1.2 ml of phosphate buffer were then added. The mixture was incubated at 30°C for 90 minutes with vigorous agitation on a vortex mixer every five minutes.

The activation reaction was stopped by adding 0.1M glycine (120 µl). The actual T3-XO coupling was performed by mixing 100 µg of XO equilibrated in phosphate buffer (200 µg) with 150 µl of the activated T3 solution from above. Incubation was performed for 30 minutes at 30°C, and the reaction was then stopped by adding 0.1M mercaptoethylamine in phosphate buffer.

The reaction medium was then purified on an Ultrogel Aca 34 (Industrie Biologique Francaise) column with 0.1M phosphate buffer, pH 7, containing 0.2% bovine serum albumin (BSA) as eluant. The XO enzyme activities were measured for each fraction. The fractions containing the coupling derivative, T3-XO, were localized after binding the latter to tubes coated with anti-T3 antibodies prepared using IgG obtained in rabbits. After addition to each coated tube of 100 µl of fraction diluted in 0.1M barbital buffer, pH 8.6, containing 0.1% of BSA, incubation was performed for one hour at 37°. The tubes were washed with distilled water and the immobilized XO activities determined by adding 200 µl of signal reagent.

Figure 10 shows the results of the binding to the anti-T3 solid phase after the passage of the coupling medium through an Ultrogel Aca 34 column. An immuno-reactive peak was localized and the corresponding fractions were combined.

Legend of Figure 10

X _____ X total XO activity
_____ XO activity bound to the Anti-T3
solid phase

5

b) Immunoassay of T3 by a Competitive
Binding Procedure

10

The tracer obtained above was used to carry out an immunoassay of T3. Figure 11 shows the corresponding calibration curve.

Legend of Figure 11

15

o _____ o counting for 1 second performed 15
minutes after the start of the
reaction

20

x _____ x counting for 1 second performed 16
hours after the start of the
reaction

Abscissa: pmol of T3 (logarithmic scale)

25

Ordinate: counts per second $\times 10^{-2}$ (linear scale)

30 The assay employed tubes coated with anti-T3 IgG described above. The incubation medium consisted of variable concentrations of T3 between 10 and 0.1 pmol in a volume of 100 μ l in the presence of the tracer (100 μ l).

35 All the dilutions were performed in 0.1M barbital buffer, pH 8.6, containing 0.1% of BSA. The incubation period at 37° was one hour. The determination

of the immobilized XO activity was performed as described above.

5 The amount of light generated by the XO-mediated chemiluminescent reaction after 16 hours of reaction demonstrated only a small decrease in the light output when compared to the light output measured after 15 minutes of reaction. Stability of the XO-dependent chemiluminescent reaction, both in the solid phase and in the
10 liquid phase, may hence be inferred.

Example 8: Detection of a biotinylated lambda DNA immobilized on a solid support.

15 Lambda phage DNA was biotinylated by nick translation using commercially available reagents (ENZO BIOCHEMICALS, N.Y., U.S.A.). The obtained preparation containing 1% biotin (w/w) was spotted on a nylon membrane (0 ng/2 μ l, 2 ng/2 μ l, 20 ng/2 μ l, 200 ng/2 μ l),
20 denaturated, in situ neutralized, and then irreversibly immobilized by ultraviolet irradiation (254 nm). The detection of this immobilized biotinylated DNA was performed as follows:

- 25 - The membranes were saturated with PBS containing 3% BSA for at least one hour at ambient temperature;
- 30 - A solution of the preformed complex biotinylated XO (5 μ g/ml) - Streptavidin (0.5 μ g/ml) in PBS containing 3% BSA was transferred to the membranes and incubated for two hours at ambient temperature;

- The membranes were washed twice in PBS, containing 3% BSA and 0.05% Triton X 100 (v/v) for 10 minutes at ambient temperature;
- 5 - The spots corresponding to the various doses of biotinylated lambda DNA were cut and placed in signal reagent (0.5 ml);
- 10 - The tubes were counted in the luminometer for five seconds.

The following results were obtained:

15	Biotinylated DNA	Counts Per Second
	<u>(ng/ul)</u>	
	0	6100
	2	22436
	20	53480
20	200	81445

It was thus demonstrated that the XO dependent luminescent response is related to the amount of biotinylated lambda DNA, immobilized on a solid support.

25

The foregoing description of the invention has been directed to particular embodiments for purposes of explanation and illustration. It will be apparent, however, to those skilled in the art that many modifications and changes in the processes of preparing and
30 implementing the described embodiments can be made without departing from the essence of the invention. Applicant intends in the following claims to cover all equivalent modifications and variations as fall within the scope of
35 the claims.

CLAIMS

1. A signal reagent for producing long term light emission useful in chemiluminescent assay comprising oxidase , an oxidase substrate, a chemiluminescent reagent and an enhancer at a pH ranging from about 9.5 to about 10.5.
- 5 2. A signal reagent of claim 1 wherein the oxidase is xanthine oxidase, choline oxidase, fumarate oxidase or sarcosine oxidase.
3. A signal reagent of claim 1 wherein this oxidase is xanthine oxidase.
- 10 4. The signal reagent of claim 3 wherein the xanthine oxidase substrate is xanthine, hypoxanthine, acetaldehyde or purine.
5. The signal reagent of claim 3 wherein the xanthine oxidase substrate is hypoxanthine.
- 15 6. The signal reagent of claim 1 wherein the chemiluminescent reagent is luminol.
7. The signal reagent of claim 1 wherein the enhancer is a transition metal complex.
8. The signal reagent of claim 1 comprising xanthine
20 oxidase, hypoxanthine, luminol, and iron EDTA complex enhancer dissolved in carbonate, barbitol, borate, phosphate or TRIS buffer or other similar buffers providing a pH ranging from about 9.5 to about 10.5.
- 25 9. A method for a competitive chemiluminescent assay of an analyte, resulting in a long term light emission, comprising the step of using a signal reagent according to anyone of claims 1 to 8.
10. The method of claim 9 wherein the analyte to be assayed is a hapten, or an antigen.
- 30 11. The method of claim 10 wherein the analyte is triiodothyronine.
12. The method of claim 9 wherein the ligand is an antibody.
13. The method of anyone of claims 9 to 12 wherein

the tracer consists of an oxidase coupled to the analyte through a direct covalent linkage or through a biotin, avidin linkage.

14. The method of anyone of claims 9 to 13 wherein
5 the ligand is coated onto a solid support by reacting it with a specific binding partner to the ligand which has first been coated to solid support.

15. A method for a sandwich chemiluminescent assay or immunoblotting of an analyte, resulting in a long term
10 light emission, comprising the step of using a signal reagent according anyone of claims 1 to 8.

16. The method of claim 15 wherein the analyte to be assayed is an antigen, or antibody.

17. The method of claim 16 wherein the analyte is
15 prolactin, antitoxoplasma,^{or} antichlamydia antibody.

18. The method of claim 15 wherein the ligand is a monoclonal antibody.

19. The method of claim 15 wherein the tracer consists of an oxidase coupled to the complementary ligand through
20 a direct covalent linkage or through a biotin, avidin linkage.

20. A method for the immunoassay of an oxidase, such as xanthine oxidase, resulting in a long term light emission, comprising the steps of :

- 25 a) providing a solid support coated with an antibody to said oxidase;
- b) incubating the sample containing the oxidase to be assayed in the presence of the solid support;
- c) washing the solid support;
- 30 d) adding to the solid support a signal reagent according to anyone of claims 1 to 8.
- e) measuring the chemiluminescent response and deducing the amount of oxidase in the sample.

21. A chemiluminescent method for detecting an
35 oligonucleotide analyte, resulting in a long term light emis-

sion, comprising the step of using a signal reagent according to anyone of claims 1 to 8.

22. A chemiluminescent method for detecting an oligonucleotide analyte, resulting in a long term light emission, comprising the steps of :

- a) depositing in single stranded form the oligonucleotide analyte on a solid support;
- b) incubating under hybridizing conditions the oligonucleotide analyte of step a) with a tracer consisting of a coupling product of an oxidase and a ligand which is a complementary oligonucleotide binding partner to the analyte;
- c) washing the solid support;
- d) adding to the washed support a signal reagent according to anyone of claims 1 to 8.
- e) measuring the chemiluminescent response and deducing the presence of the analyte.

23. The method of claim 22 wherein the tracer consists of an oxidase coupled to the ligand through a direct covalent linkage or through a biotin, avidin linkage.

24. A kit for use in a competitive or sandwich chemiluminescent assay for the detection of an analyte, resulting in a long term light emission, the kit comprising the following reagents in separate containers :

- a) a solid support coated with a ligand which is a specific binding partner of the analyte;
- b) a tracer consisting of a coupling product of an oxidase and either of the analyte or a ligand; and
- c) a signal reagent according to anyone of claims 1 to 8.

25. The kit of claim 24 wherein the tracer consists of an oxidase coupled to either the analyte or ligand through a direct covalent linkage or through a biotin, avidin linkage.

26. A kit for use in chemiluminescent assay to detect an oligonucleotide analyte resulting in a long term light emission, comprising the following reagents in separate containers :

- 5 a) a solid support for depositing the analyte ;
- b) a tracer consisting of a coupling product of
 an oxidase and a ligand which is a complemen-
 tary nucleotide binding partner to the ana-
 lyte; and
- 10 c) a signal reagent according to anyone of claims
 1 to 8.

27. The kit of claim 26 wherein the tracer consists of xanthine oxidase coupled to the analyte through a direct covalent linkage or through a biotin, avidin linkage.

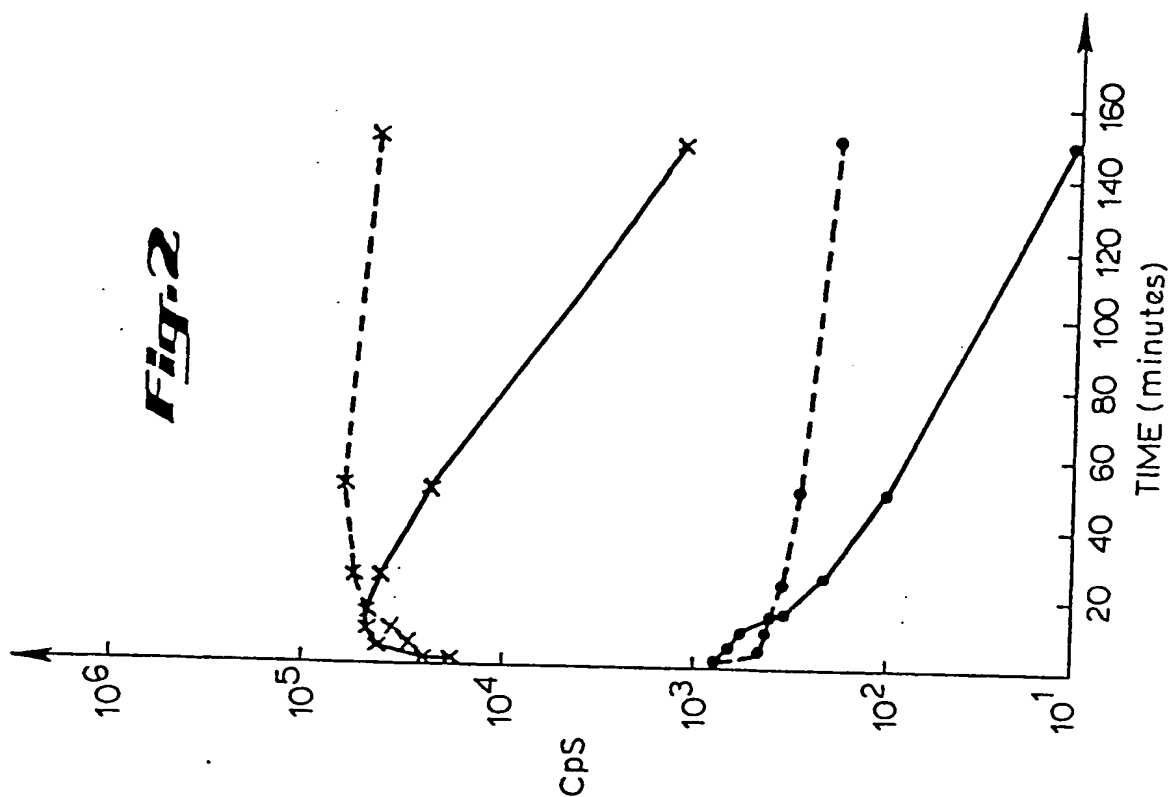
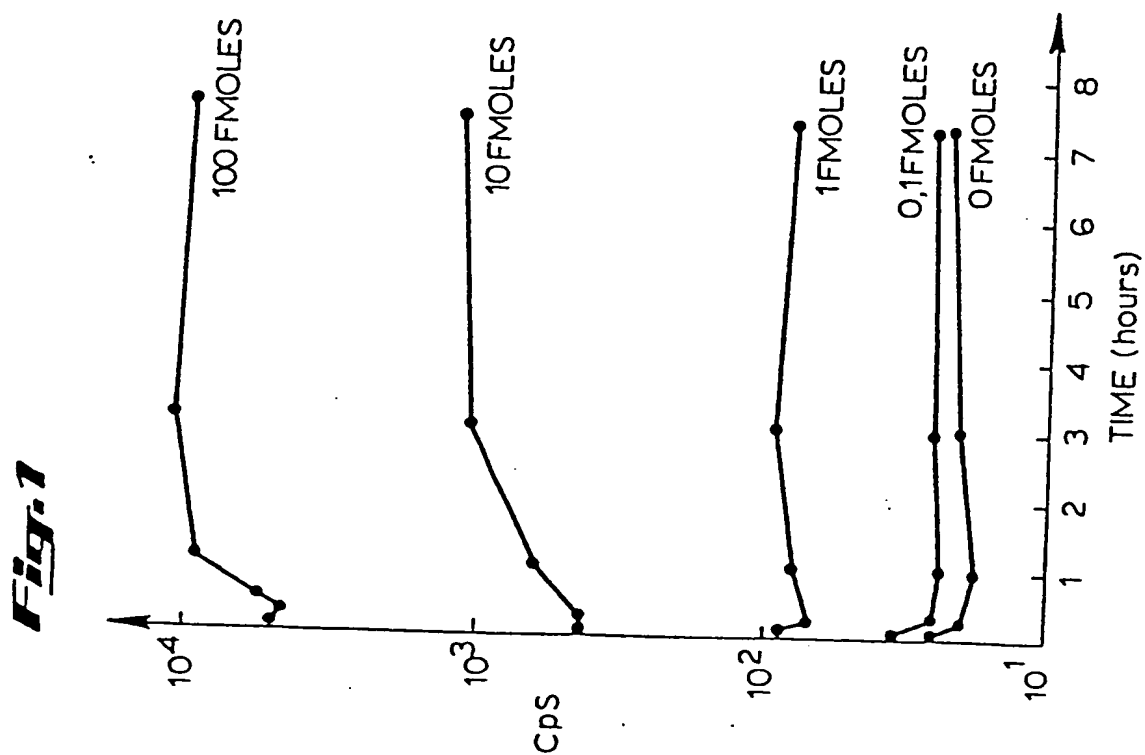
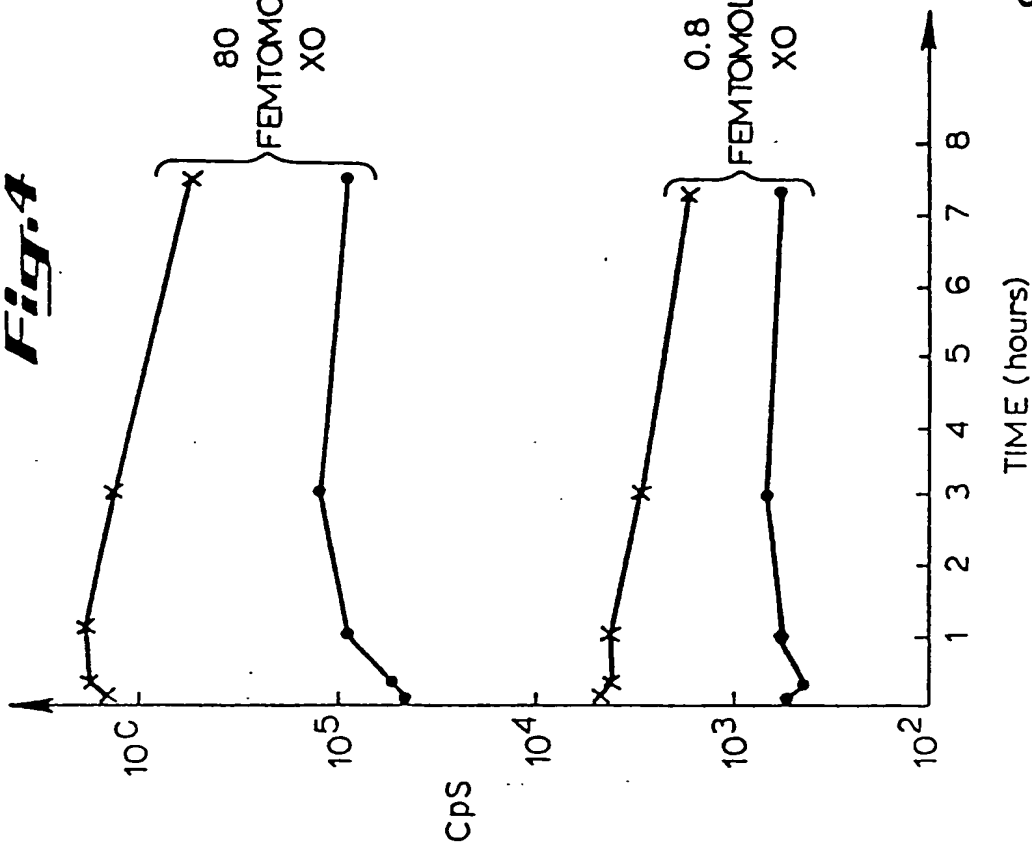
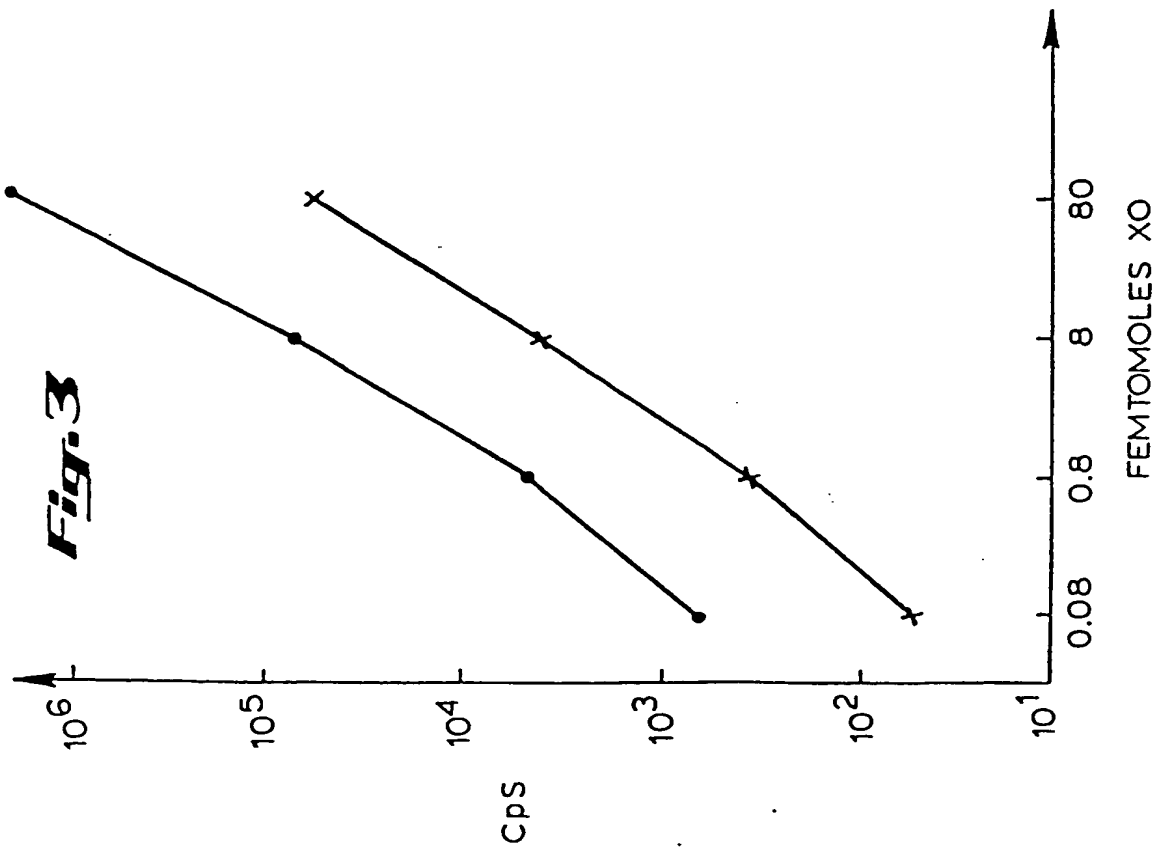
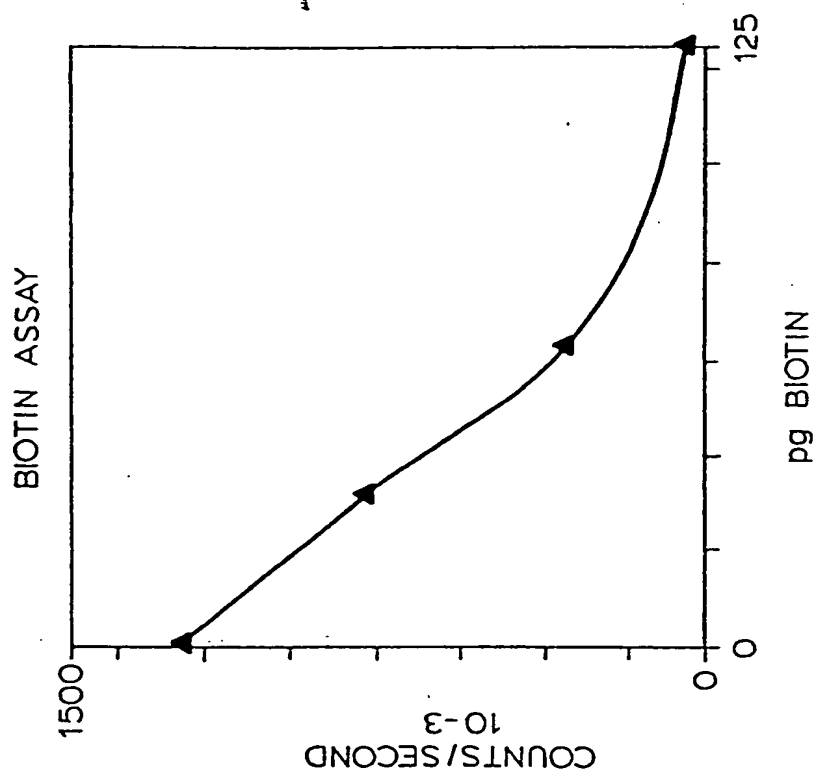
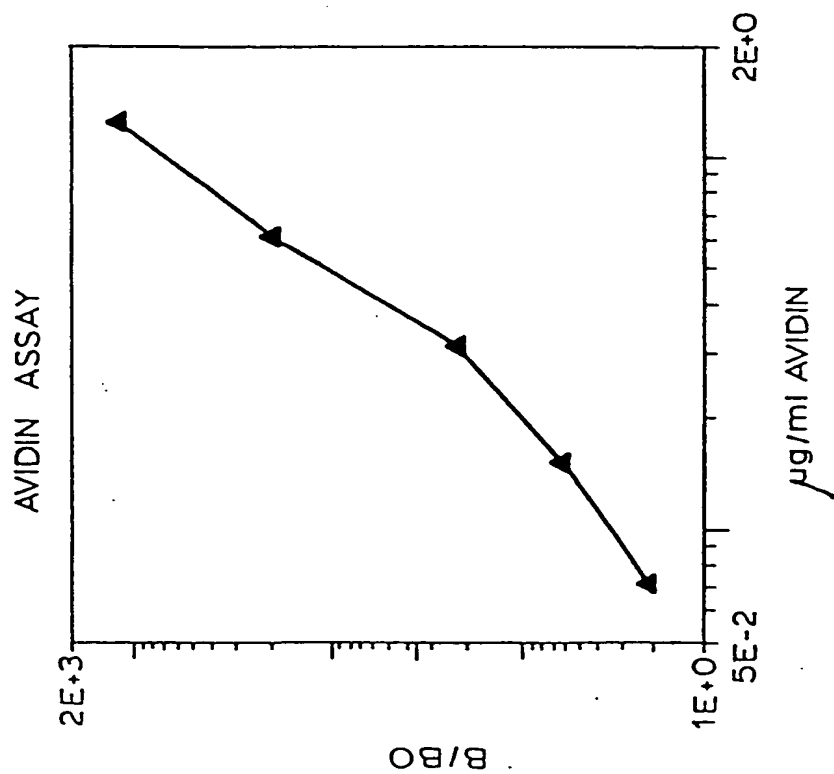


Fig. 4**Fig. 3**

**Fig-6****Fig-5**

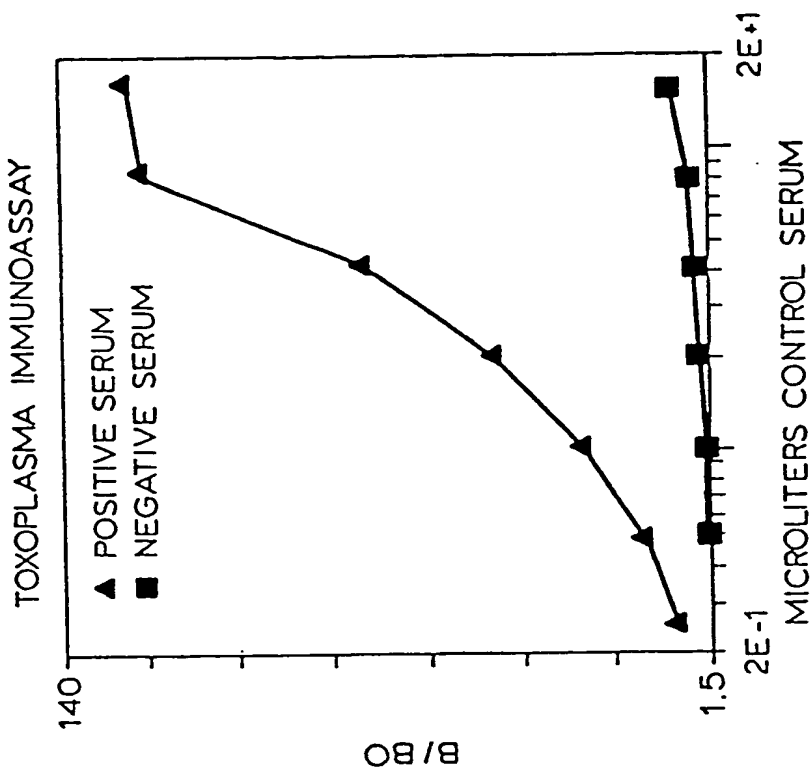


Fig. 8

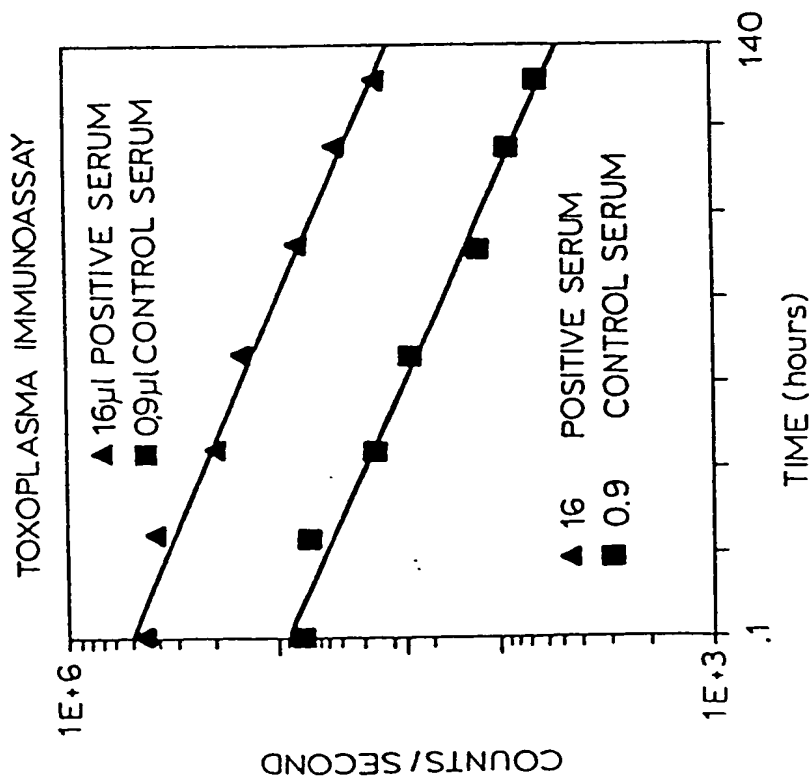


Fig. 7

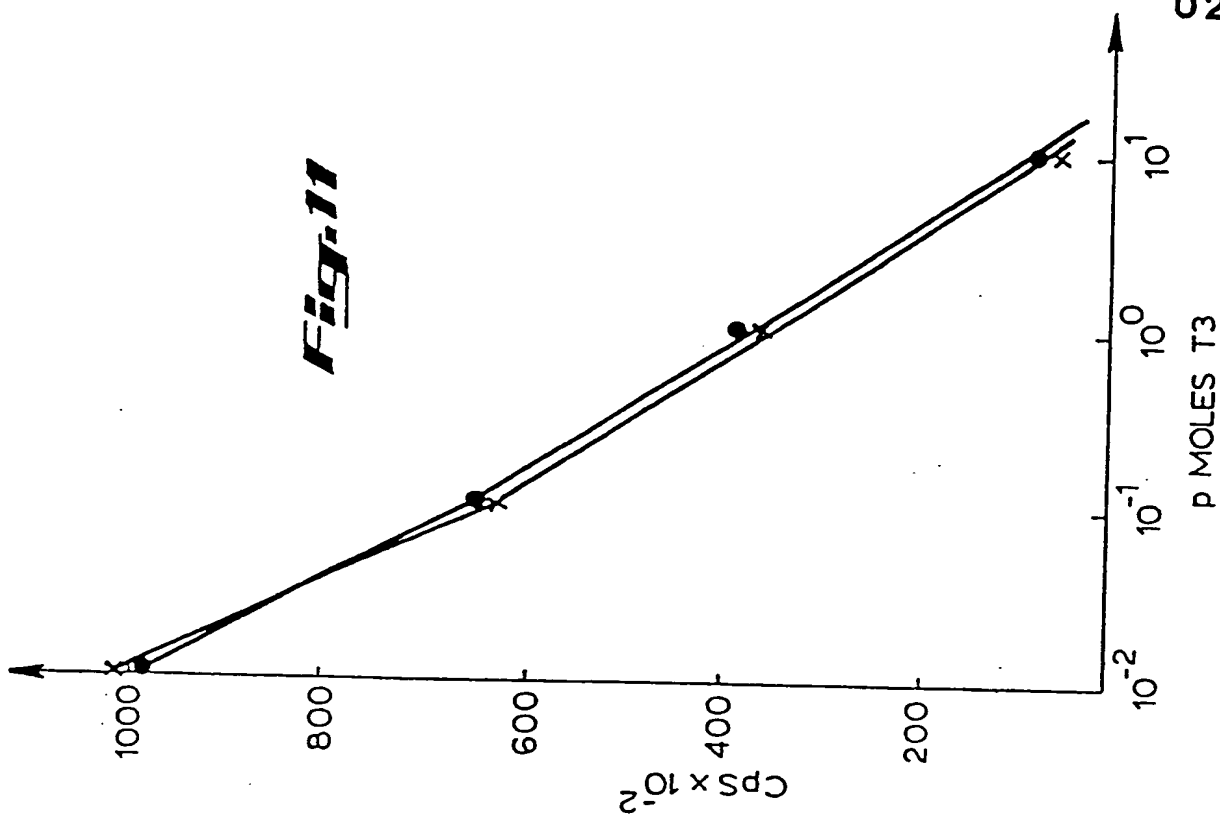
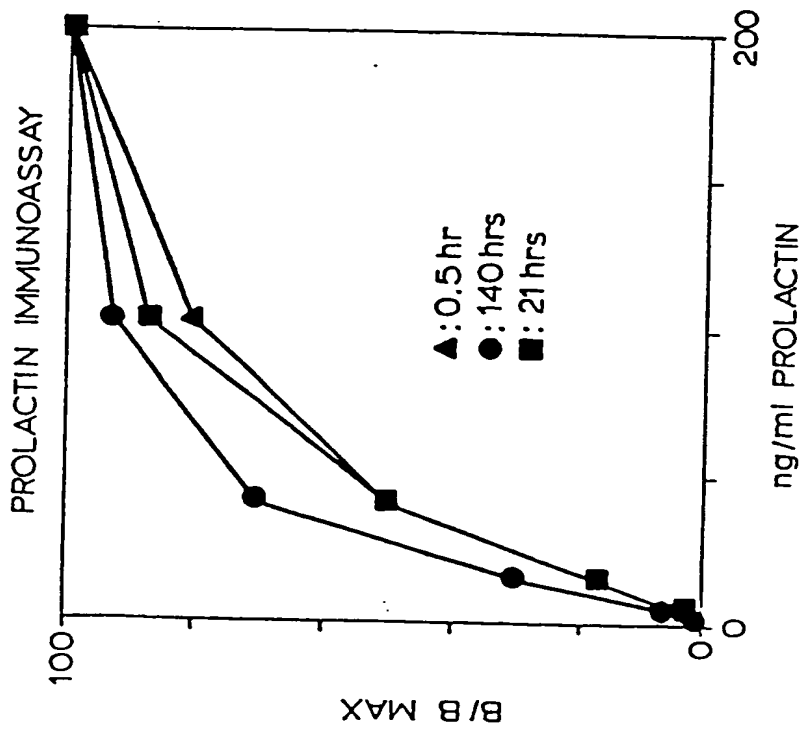
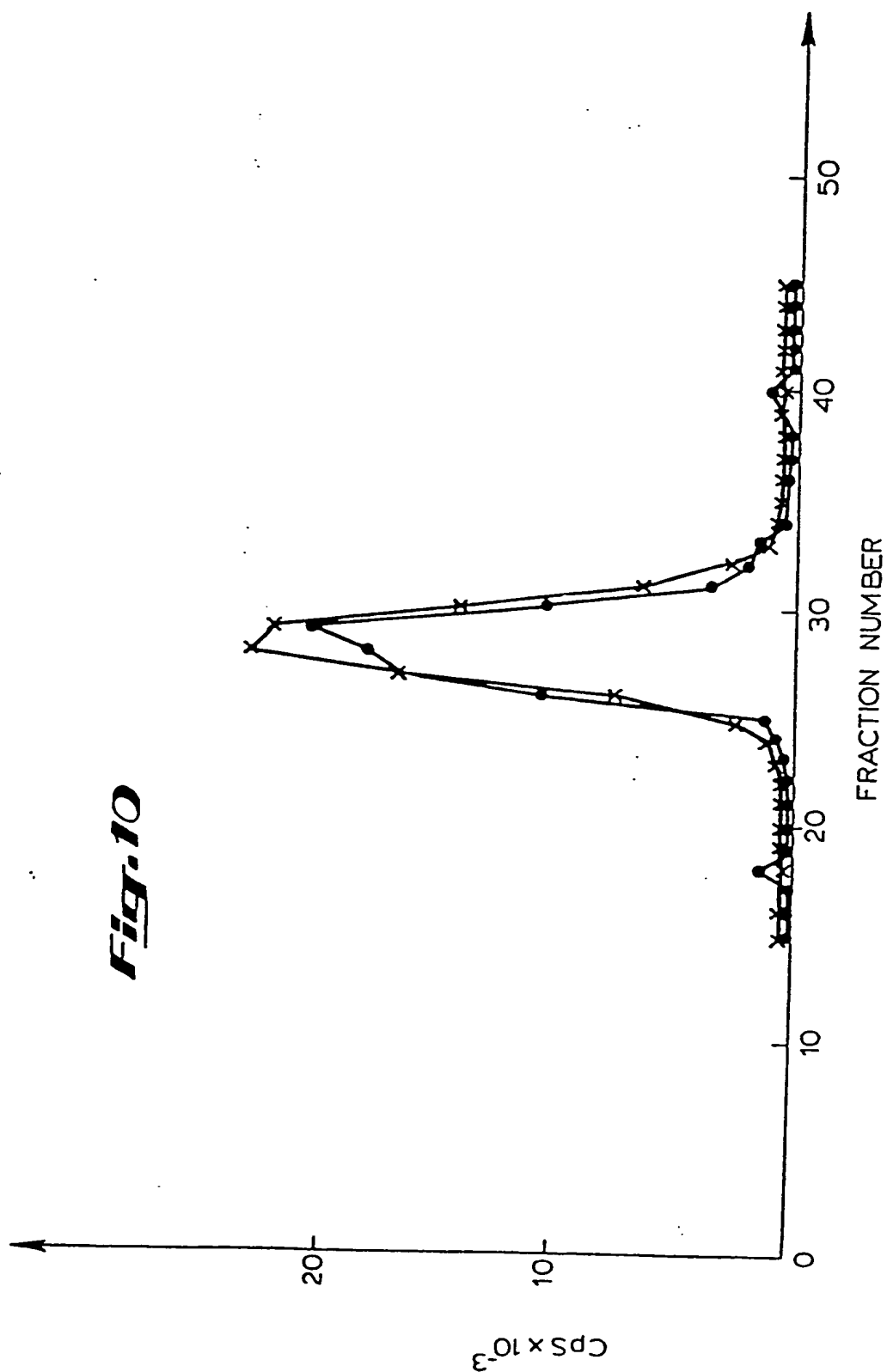
Fig. 11**Fig. 9**

Fig. 10

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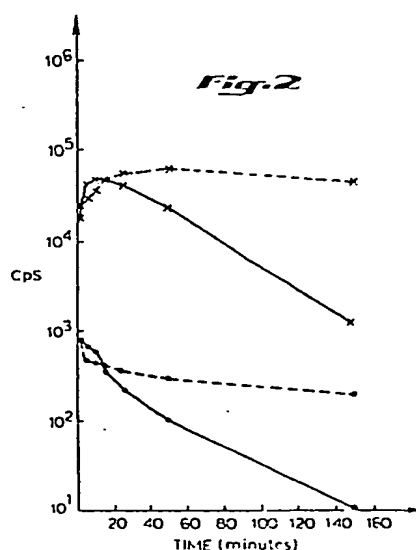
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(54) Use of oxidase enzyme systems in chemiluminescent assays.

(57) An oxidase enzyme system to provide long lived entities capable of being recognized by a chemiluminescent reagent is disclosed. In the preferred embodiments, a specific binding pair ligand or analyte is coupled with xanthine oxidase, either directly or via a streptavidin bridge. Thereafter the presence of an analyte can be determined by a chemiluminescent emission upon addition of a signal reagent comprising an oxidase substrate, a chemiluminescent reagent, such as luminol, and an enhancer, i.e. iron EDTA complex with a buffer providing a pH ranging from about 9.5 to about 10.5. The resulting chemiluminescent signal is stable and detectable for many hours or days after initiation. The chemiluminescent oxidase system is particularly useful for immunoassays and nucleotide probe analysis.





European Patent
Office

EUROPEAN SEARCH REPORT

0256932

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	WO-A-8 503 356 (MOLECULAR BIOSYSTEMS INC.) * Pages 11-17; claims 1,10,11,12,27-31,36,40,42,51,53,54,69 *	1-3,6	C 12 Q 1/26 G 01 N 33/535 G 01 N 33/573 C 12 Q 1/68 G 01 N 33/78
X	EP-A-0 016 845 (KYOWA HAKKO KOGYO CO. LTD) * Abstract; claims 1,3,9,12 *	1-5	
X	ANALYTICAL CHEMISTRY, vol. 50, no. 8, July 1978, pages 1114-1120, American Chemical Society, Washington, US; H.R. SCHROEDER et al.: "Chemiluminescence yields and detection limits of some isoluminol derivatives in various oxidation systems" * Page 1115, column 2; page 1117, column 1; page 1118, tabel II *	1-6	
A	EP-A-0 175 560 (E.I. DU PONT DE NEMOURS) * Claims 1,2,10 *	1,3,24	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
A	US-A-4 376 825 (K.E. RUBINSTEIN) * Abstract; column 33, line 68; column 45, lines 1-5 *	1-5,10,11	C 12 Q 1/00 G 01 N 33/00
A	CHEMICAL ABSTRACTS, vol. 97, no. 21, 22nd November 1982, pages 324-325, abstract no. 177149j, Columbus, Ohio, US; M. TIEN et al.: "The multiple effects of ethylenediaminetetra-acetate in several model lipid peroxidation systems", & ARCH. BIOCHEM. BIOPHYS. 1982, 218(2), 450-8	1,8	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 14-04-1988	Examiner MEYLAERTS H.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			